Isolation and reconstitution of the *N*-formylpeptide receptor from HL-60 derived neutrophils

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A multifunctional receptor for *N*-formylpeptides exists on the membranes of neutrophils. This receptor has now been isolated from neutrophils derived from HL-60 promyelocytic leukemia cells. After solubilization by Nonidet-P40 and purification by affinity chromatography and HPLC the isolated receptor was reconstituted into egg phosphatidylcholine vesicles by SM-2 Bio-Bead removal of the Nonidet-P40. Analysis of the affinity and selectivity of the receptor was done by direct binding of two high-affinity ligands, formyl-Met-Leu-[^{3}H]Phe-OH and formyl-Nle-Leu-Phe-[^{3}H]Tyr-OH. The data suggest that the receptor can be isolated and reconstituted without apparent alteration of its binding affinity and selectivity, and that there appear to be no co-factors or subunits upon which these binding characteristics are dependent.

Receptor *Solubilization* *Reconstitution* *Lipid vesicle* *Affinity chromatography* *Scatchard analysis*

1. INTRODUCTION

A multifunctional receptor for low-M_r chemotactic oligopeptides, of which *N*-formylmethionylleucylphenylalanine-OH (FMLP) is the prototype, exists on the membrane of mammalian phagocytes [1–3]. These peptides stimulate chemotaxis, lysosomal enzyme secretion and a variety of other cellular responses by interaction with this specific membrane receptor, often referred to as simply the chemotactic peptide receptor. This receptor has been solubilized from human [4,5] and rabbit [6] neutrophils and subsequently purified from human neutrophils by affinity chromatography [5]. The major protein isolated had an M_r of ~68000 and retained its high affinity for FMLP.

Neutrophils derived from HL-60 promyelocytic leukemia cells have also been shown to express the FMLP receptor [7,8]. The potential for growth of large quantities of these cells prompted us to use this source for isolation of enough receptor to attempt their reconstitution into lipid vesicles. In this paper we describe the solubilization and isolation of the chemotactic receptor from HL-60 derived neutrophils, and the subsequent reconstitution of the receptor into egg phosphatidylcholine (PC) vesicles.

2. EXPERIMENTAL

HL-60 promyelocytic leukemia cells were roller cultured in 850-cm^2 roller bottles (1 rpm) in media consisting of RPMI 1640, 10% fetal calf serum, 5% glutamine, and 50000 units/l of penicillin–streptomycin. Bottles were seeded at 4×10^5 cells/ml and stimulated with 1.25% dimethyl sulfoxide and 10^{-5} M dexamethasone. After 7 days the cells were at a density of approx. 3×10^6 cells/ml, with >95% differentiated into band cells and metamylocytes. Approx. 2×10^9 cells were collected and suspended in 0.1 M NaCl, 10 mM Hepes and 2.5 mM $MgCl_2$ (pH 7.2) and disrupted by N_2 cavitation [9]. The crude membrane fractions consisting of both the 4×10^5 $g \cdot$min and 9×10^6 $g \cdot$min pellets were pooled, resuspended in

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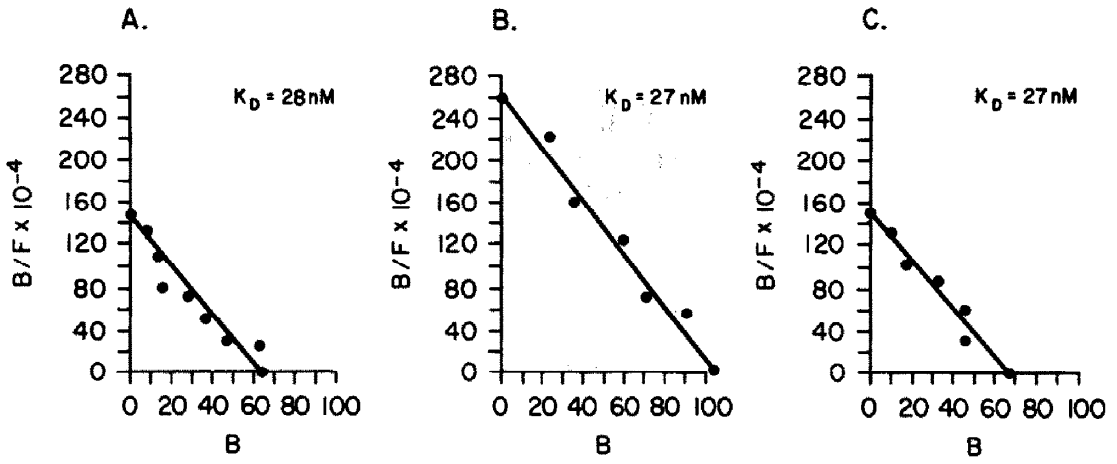

Fig.1. Scatchard plots for FML[^{3}H]P binding to: (A) intact HLC-60 neutrophils, (B) reconstituted affinity column eluate, and (C) reconstituted 66 kDa peak. Abscissa: (A) fmol specific bound per 10^6 cells, (B and C) fmol specific bound per tube.

0.1 M NaCl, 10 mM Hepes (pH 7.2) to about 1 mg/ml and solubilized with 0.6% Nonidet-P40. The solubilized solution was passed twice over a 2 ml AH Sepharose 4B affinity column coupled to FMLP (10–15 μmol/g dried resin as determined by amino acid analysis), washed with 20 ml of solubilization buffer, and eluted with another 6 ml of buffer containing 20 mg FMLP (dissolved in 200 μl dimethyl sulfoxide). The receptor-rich eluate was simultaneously dialyzed and concentrated to about 0.5 ml by vacuum ultrafiltration across a dialysis sac (M_r cut-off 14000) in 0.1 M NaCl, 10 mM Hepes and 0.03 ml Nonidet-P40/l (pH 7.2). The concentrate was then chromatographed in the same buffer by HPLC on a calibrated TSK 4000 sizing column, and the 66 kDa peak collected and reconcentrated by vacuum ultrafiltration.

Table 1

Affinity of FMLP and FNLPT for in situ and reconstituted receptors

Preparation	K_d (nM)	
	FMLP	FNLPT
Intact cells	28	5
Reconstituted affinity column eluate	27	9
Reconstituted 66 kDa peak from HPLC	27	10

Formylmethionylleucylphenylalanine (FMLP) and formylnorleucylleucylphenylalanyltyrosine (FNLPT)

Egg PC (2 mg/ml) was dried under N_2, resuspended in 0.1 M NaCl, 10 mM Hepes and 1 mM $CaCl_2$ (pH 7.2) and solubilized to absolute clarity with Nonidet-P40. Approx. 0.7 μg/ml of protein from the concentrated 66 kDa fraction, or 1.6 μg/ml of protein from the concentrated affinity column eluate were added to the solubilized PC solution, followed by 0.5 g/ml SM-2 Bio-Beads. The Bio-Beads were prepared as in [10] and the vesicles prepared according to [11].

Direct binding to the resulting receptor containing PC vesicles was evaluated by a standard filtration assay [12] using two high-affinity ligands, FML[^{3}H]P and formyl-Nle-Leu-Phe-[^{3}H]Tyr-OH [FNLP[^{3}H]T]. Data were analyzed as in [13].

Protein was estimated from the chromatograms using bovine albumin as the standard. The detector was set at 246 nm which shows sufficient sensitivity for protein while minimizing the absorbance of Nonidet-P40.

3. RESULTS

Our early solubilization studies showed that Nonidet-P40, CHAPS, and digitonin, but not β-octylglucopyranoside, could be used to solubilize

the chemotactic receptor from HL-60 neutrophils. Equilibrium dialysis binding analysis of the preparations showed unaltered affinities for FMLP, but due to the difficulty in working with digitonin, and the receptor's apparent instability in CHAPS, Nonidet-P40 was chosen for further studies. The solubilized receptor was stable for over a week when stored at 4°C, but was inactivated when freeze-thawed. The stability of the reconstituted receptor followed the same pattern.

Fractionation of the concentrated affinity column eluate by HPLC on a TSK 4000 sizing column showed a large predominating 66 kDa peak and two smaller peaks of approx. 130 kDa and 250 kDa. When the 66 kDa peak was collected, reconcentrated and rechromatographed it produced a single symmetrical peak. Typically, 1–2 μg of protein were obtained from 2×10^9 cells.

There was no significant change in the affinity and selectivity of the reconstituted receptor as determined by direct binding of the two high-affinity ligands (table 1). Scatchard analysis of the binding data indicates a single population of sites for FMLP (fig.1) and FNLPT (not shown). To determine if the receptor's binding characteristics were dependent upon or altered by a co-factor present in the intact cell or a subunit which coeluted from the affinity column, the K_d values were determined for the reconstituted affinity column eluate as well as for the isolated 66 kDa peak. There were no significant differences between the two (table 1).

4. DISCUSSION

The Nonidet-P40 solubilized chemotactic receptor from HL-60 derived neutrophils has been isolated and successfully reconstituted into PC vesicles with no apparent loss of binding affinity or selectivity. The receptor from these cells isolates as a 66 kDa protein, which is in agreement with that previously determined for the receptor isolated from human neutrophils [5]. The receptor appears to be quite stable in both its solubilized and reconstituted forms, thus facilitating its isolation and handling.

Although it cannot be stated with certainty, the retention of affinity and selectivity throughout the isolation procedures suggests that there are no essential co-factors upon which binding of FMLP is dependent. Furthermore, the use of the HL-60 cell line is a distinct advantage in that development and optimization of isolation and reconstitution procedures are greatly facilitated by an abundant starting material. Retention of binding affinity through both the affinity chromatography and HPLC purification steps indicates that the receptor is not absolutely dependent upon native lipid for its successful reconstitution or ligand binding.

Finally, it must be realized that the functionality of this isolated receptor cannot be inferred from the retention of binding characteristics. The measurement of functionality of reconstituted receptors has been achieved only with the nicotinic receptor [14] and recently in a coupled assay with solubilized (but not isolated) β-adrenergic receptor [15]. The measurement of a functional response of the reconstituted FMLP receptor remains an important goal.

In summary, the FMLP receptor of HL-60 derived neutrophils has been isolated and reconstituted. This, we believe, offers a unique opportunity to study this important receptor, as well as providing a well defined system to study ligand–receptor interactions in general.

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